15413/83 COMMONWEALTH of AUSTRALIA

PATENTS ACT 1952

LODGED AUSTRALIA 10 JUN 1982

SECTION 34(4)(a) DIRECTION AS TO NOMINATION OF STANDARD PATENT

NAME DIRECTED: HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND MEDICINE, UNIVERSITY OF MELBOURNE, PARKVILLE, VICTORIA, COMMONWEALTH OF AUSTRALIA.

AUSTRALIAN PATENT OFFICE 10 JUN 1982 Sec 34(4)

561670

We, GEOFFREY WILLIAM TREGEAR, of 62 Hawthorn Grove, Hawthorn, in the State of Victoria, Australia, DU YU-CANG, of Shanghai Institute of Biochemistry, Academica Sinica, Shanghai, The People's Republic of China and HUGH DAVID NIALL, of 3 Bendigo Avenue, Elwood, in the State of Victoria, Australia

COMPLETE AFTER PROVISIONAL SPECIFICATION No. 15413/83

hereby apply for the grant of a Standard Patent for an invention entitled:

"PEPTIDES WITH RELAXIN ACTIVITY"

which is described in the accompanying provisional specification.

~~Details of basic application(s):~~

~~Number~~ ~~Convention Country~~ Date

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 23.3.87

PATENT OFFICE 10 TEN DOLLARS

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RECEIVED Date 17.6.82 P1 4385

The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

Dated this 10th day of June 19 82

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To: THE COMMISSIONER OF PATENTS

H. W. Rimington

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Davies & Collison, Melbourne and Canberra.

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT

Insert title of invention.

In support of the Application made for a patent for an invention entitled: "PEPTIDES WITH RELAXIN ACTIVITY"

Insert full name(s) and address(es) of Declarant(s) being the applicant(s) or person(s) authorized to sign on behalf of an applicant company.

I ROBERT HOOKHAM, Business Manager & Secretary, ~~We~~ of Howard Florey Institute of Experimental Physiology and Medicine, a body corporate established under the laws of the State of Victoria, of the University of Melbourne, Parkville, in the State of Victoria, Commonwealth of Australia,

do solemnly and sincerely declare as follows :-

Cross out whichever of paragraphs 1(a) or 1(b) does not apply.

1. ~~(a)~~ ~~I am~~ / We are ~~the applicant..... for the patent~~

1(a) relates to application made by individual(s).

or (b) I am authorized by HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND MEDICINE

1(b) relates to application made by company; insert name of applicant company.

the applicant..... for the patent to make this declaration on ~~its~~ / their behalf.

Cross out whichever of paragraphs 2(a) or 2(b) does not apply.

2. ~~(a)~~ ~~I am~~ / We are ~~the actual inventor..... of the invention~~

2(a) relates to application made by inventor(s)

or (b) GEOFFREY WILLIAM TREGEAR, of 62 Hawthorn Grove, Hawthorn, Victoria 3122, Commonwealth of Australia;
DU YU-CANG, of Shanghai Institute of Biochemistry, Academica Sinica, Shanghai, The People's Republic of China; and
HUGH DAVID NIALL, of 3 Bendigo Avenue, Elwood, Victoria 3184, Commonwealth of Australia

2(b) relates to application made by company(s) or person(s) who are not inventor(s); insert full name(s) and address(es) of inventors.

~~is~~ / are the actual inventor...s..... of the invention and the facts upon which the applicant..... ~~is~~ / are entitled to make the application are as follows :- the said actual inventors assigned their right in the invention to Relaxin Pty. Ltd., who in turn assigned the rights to Howard Florey Institute of Experimental Physiology and Medicine.

State manner in which applicant(s) derive title from inventor(s).

Cross out paragraphs 3 and 4 for non-convention applications. For convention applications insert basic country(s) followed by date(s) and basic applicant(s).

3. The basic application..... as defined by Section 141 of the Act ~~was~~ / were made

in ..... on the .....

by .....

in ..... on the .....

by .....

in ..... on the .....

by .....

4 The basic application..... referred to in paragraph 3 of this Declaration ~~was~~ / were the first application..... made in a Convention country in respect of the invention the subject of the application.

Insert place and date of signature.

Declared at MELBOURNE this THIRTEENTH day of JUNE, 1986

Signature of Declarant(s) (no attestation required).

.....

.....

Note: Initial all alterations.

DAVIES & COLLISON, MELBOURNE and CANBERRA.

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(54) PEPTIDES WITH RELAXIN ACTIVITY

(71) HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND MEDICINE

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(74) DM

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(57) Claim

1. A method for the synthesis of relaxin or modified forms or analogues thereof, which comprises the steps of reducing a mixed solution of the S-sulfonated A and B peptide chains, or modified forms or analogues thereof; precipitating the reduced peptides with a non-reactive, neutral, organic, water-miscible solvents, washing the mixed peptides; oxidizing the mixed peptides in the presence of a non-reactive, water soluble, inorganic salt; and isolating the relaxin thus produced.

9. A synthetic porcine relaxin analogue consisting of the A(1-22) and B(1-25) peptide chains.

22. A porcine relaxin analogue which has been modified by one or more of the following procedures:
(a) the addition of one or more amino-acids to one or
both of the natural A and B chains;
(b) the deletion of one or more amino acids from the natural B and/or A peptide chains; .../2

(c) the chemical modification of one or more amino acids

(d) the replacement of at least one of the natural amino-acids in one or both of the A and B chains with a different amino acid of the natural B and/or A peptide chains;

with the proviso that the thus modified porcine relaxin analogues possess porcine relaxin activity.

561670

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(Original)

FOR OFFICE USE

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Actual Inventor(s): GEOFFREY WILLIAM TREGEAR
DU YU-CANG
HUGH DAVID NIALL

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PATENT OFFICE AUSTRALIAN Sec 34(4)

LODGED AT SUB-OFFICE
- 6 JUN 1983
Melbourne

Complete specification for the invention entitled:

"PEPTIDES WITH RELAXIN ACTIVITY"

The following statement is a full description of this invention, including the best method of performing it known to us :-

This invention relates to the synthesis of peptides with relaxin activity. The invention is particularly concerned with the synthesis of porcine relaxin and some structural analogues.

The ovarian peptide hormone relaxin plays an important role in pregnancy and parturition. The main biological action of the hormone is to soften and lengthen the inter-pubic ligaments: it also dilates the cervix and inhibits contractions of the uterus.

Relevant prior art is contained in the references cited herein which are identified by numbers in brackets in the text and are collected together at the end of this description. Other prior art is collected and described in the specification of British Patent No. 2,072,680 (equivalent to U.S. Serial No. 134,390, filed March 27, 1980). The content of these references is hereby incorporated into this specification.

The complete amino acid sequence of relaxin extracted from the ovaries of the pregnant pig has been recently established (1, 2) and is shown in Figure 1 of the accompanying drawings. The amino acid sequence of rat relaxin has also been elucidated (3, 4) and Gowan et al. (5) have reported a partial sequence analysis of the hormone from the shark ovary.

The general structural features of the relaxin molecule bear striking resemblance to insulin. Relaxin is composed of two peptide chains (A and B) joined by disulfide bonds through the cysteines at A9-B10 and A22-B22 (see Figure 1), with an intra-chain disulfide bridge within the A-chain between A8 and A13. The disposition of the disulfide bonds is thus analogous to that of insulin. A model of the relaxin sequence can be accommodated without strain into the three-dimensional coordinates of the insulin structure (6, 7) and circular dichroism analyses of the hormones in solution are very similar (8, 9). Thus relaxin and insulin may share common features of secondary and tertiary structure even though the hormones have no measurable overlap in biological or immunological properties (9).

Three forms of relaxin of equivalent biological activity, which are characterized by their elution behaviour on carboxymethyl cellulose chromatography, have been isolated from the ovaries of the pregnant pig. These forms of relaxin have been designated CMB, CMa' and CMa by Sherwood & O'Bryne (10). Sequence analysis has revealed that these three forms of pig relaxin have an identical A chain of 22 amino acids but differ in sequence at the carboxyl terminus of the B chain (12). CMa relaxin has a B-chain of 31 amino acids; the B-chains of CMa' and CMB are shorter, having 29 and 28 residues respectively (see Figure 1).

The predominant form of the hormone stored in the pig ovary is the CMa or B31 variant: the CMB and CMa' peptides are probably largely generated during the isolation procedures (11).

We have carried out research into the chemical synthesis of relaxin and its structural analogues as part of a systematic study of the structure-function relationships in the hormone molecule. The purified separate chains of pig relaxin have been obtained in approximately 10% yield (based on crude). The major impurities in the synthetic peptides appear to have arisen through side-reactions of the cysteine residues occurring during the HF cleavage step. In more recent syntheses improved yields have been obtained using the HF stable S-ethyl mercapto protecting group for cysteine. We have found that both the natural and synthetic B chains of pig relaxin, whether in the reduced or S-sulfonated form, are particularly difficult to dissolve in aqueous solution. In addition, the relaxin B chains exhibit unusual adsorptive properties. Column chromatography on Sephadex, cellulose, polyamide or polystyrene resins results in virtually a complete loss of peptide by irreversible absorption (12). These properties have made purification of synthetic relaxin B chain peptides and their coupling to A chain particularly difficult. Detailed studies of the conformation of the relaxin B chains by circular dichroism have revealed that the configuration of the B31, B29 and B28 peptides in solution were largely beta-structure.

We have found that conditions which give efficient combination of the insulin A and B chains (13) give very low or negligible yields when use for the structurally related relaxin. Our investigations have now shown that precipitation of the mixed relaxin peptide chains with a neutral water miscible solvent such as acetone, washing to remove reducing agent, and

the addition of a water soluble inorganic salt such as NaCl during the oxidation (recombination) step are essential requirements.

It is an object of the present invention to provide methods for the preparation of relaxin, particularly porcine relaxin and its structural analogues which provide improved product yields over the known art.

More specifically, one object of the invention is to provide a method for joining the A and B chain peptides of natural and/or synthetic porcine relaxin.

Another object is to provide a method of producing relaxin analogues by combining modified forms of the natural B and/or A relaxin chains.

A further object is to provide modifications and/or structural analogues of natural porcine relaxin.

Other aims and objects of the invention will be apparent from the following description.

In accordance with one aspect of the present invention, there is provided a method for the synthesis of relaxin or modified forms or analogues thereof, which comprises the steps of reducing a mixed solution of the S-sulfonated A and B peptide chains or modified forms or analogues thereof; precipitating the reduced peptides with a non-reactive, neutral, organic, water-miscible solvent; washing the mixed peptides; oxidizing the mixed peptides in the presence of a

non-reactive water soluble inorganic salt; and isolating the relaxin thus produced.

The above described method is particularly applicable to the synthesis of porcine relaxin, however it is also applicable to other types of relaxin, including human relaxin.

The term "non-reactive" as used throughout this description and claims is used to indicate that the atmosphere, solvent, or salt, or the like, is not chemically reactive with peptides.

The neutral, water-miscible, organic solvent used to precipitate the reduced peptides may be any such solvent known per se as a precipitant for peptides. Suitable solvents include the lower aliphatic ketones and alcohols, with acetone being the preferred solvent.

Any non-reactive, water-soluble inorganic salt may be present during the oxidation or recombination step. The use of sodium chloride is, however, preferred.

Preferably, the isolation and purification of the product is carried out using chromatographic methods.

More specifically in accordance with this aspect of the invention, a method for the synthesis of relaxin comprises the steps of:

separately preparing or isolating the S-sulfonated A and B peptide chains;

forming a mixture of the S-sulfonated A and B peptides;

reducing the mixture at pH 7 to 9, preferably 8.3, under a non-reactive atmosphere, preferably nitrogen, for at least 6 minutes;

adjusting the pH of the reduced mixture to 4.5 to 5.5, preferably pH5, preferably with acetic acid;

adding a non-reactive, neutral, water-miscible, organic solvent, preferably acetone, to the mixture to precipitate the mixed peptides;

washing the mixed peptides with a suitable solvent, preferably ethyl acetate and ether, to remove the reducing agent;

oxidizing the mixed peptides at pH 9.5 to 11, optimally at about 10.4 to 10.6,for about 48 to 72 hours at about 0° to 10°C, preferably 5°C, in the presence of a non-reactive, water soluble, inorganic salt, preferably sodium chloride, in a concentration of at least about 0.1M, preferably about 0.5 to 1M.

Throughout this specification reference will be made to the accompanying drawings in which:-

Figure 1 shows the amino-acid sequences of the A and B chains of porcine relaxin;

Figure 2 shows diagrammatically, a purification procedure for a crude synthetic S-sulfonated porcine relaxin B-chain;

Figure 3 shows circular dichroism (CD) spectra for:-

(a) native porcine relaxin;

(b) the S-sulfonated, and reduced (un-sulfonated) A chains;

(c) S-sulfonated, full-length and shortened B chains;

(d) combinations and mixtures of various A and B chains;

Figure 4 shows the results of typical purification procedures as described herein; and

Figure 5 shows the results for the final ion-exchange purification stage of a synthetic (A22 B25) combination mixture.

Using the above-described method, the natural A(1-22) and B(1-28) chains were combined in yields of up to 25% (based on the B chain). The combination yield for synthetic A(1-22) and B(1-28) chains was 0.7%.

We have also found that the combination yields of the synthetic A and B chains can be improved by shortening of the B chain at the carboxyl terminus to as little as 25 residues. As shown by Figure 3(c), the CD spectra indicate that shortening to 25 residues results in a change of structure from the beta-configuration to an unordered conformation. This conformational change is reflected in an enhanced solubility of the peptide in aqueous solution, a loss of the adsorptive behaviour and an improvement in the combination yield with the A chain. Using the

above-described method the combination yield rises to 10%. The specific activity of the A22 B25 peptide as so prepared is 33% of that of the natural A22 B31 peptide. After purification, the A22 B25 peptide has a specific activity of 93% of the native A22 B31 relaxin. As shown in Figure 3(d) the CD spectrum of the A22 B25 peptide closely resembles that of native relaxin (Figure 3(a)).

Shortening is also tolerated at the amino terminus of both the A and B chains as well as at the carboxyl terminus of the B chain. The B(4-23)NH_2 chain has a similar CD spectrum (Figure 3(c) to the B(1-25) chain. The synthetic peptide A(4-22)B(4-23)NH_2 still retains significant biological activity.

Thus in accordance with another aspect of this invention, there is provided a relaxin analogue, particularly a porcine relaxin analogue, consisting essentially of shortened forms of the natural B and/or A peptide chains.

This aspect of the invention also provides a method for producing a relaxin analogue, particularly a porcine relaxin analogue, which comprises the step of forming the shortened B and/or A peptide chains and combining them by the method of the invention as described above.

The preferred shortened porcine relaxin peptide chain combinations are A(1-22) and B(1-25).

A further aspect of the present invention provides for chemical modification of the B and/or A chains (in

either full-length or shortened form) prior to combination by the method of the invention. Two types of chemical modification may be employed, either singly or in combination.

The first type involves the modification of one or more of the amino-acids which occur in the natural B and/or A chains. Such modification will generally involve protection of active groups on one or more of the amino-acids by methods known per se, and the protecting groups may, if desired, be removed after combination of the (modified) A and B chains.

Examples of this type of modification include the acetylation or similar protection of free amino groups, amidation of C-terminal groups, or the formation of esters of hydroxyl or carboxylic groups. The formyl group is a typical example of a readily-removable protecting group.

The second type of modification includes replacement of one or more of the natural amino-acids in the B and/or A chains with a different amino acid (including the D-form of a natural amino-acid). This general type of modification may also involve the deletion of a natural amino-acid from the chain or the addition of one or more extra amino-acids to the chain.

The purpose of such modifications is to enhance the combination yields of the A and B chains, while maintaining the activity of the product, i.e., relaxin or an analogue thereof, or to enhance the activity of the product for a given combination yield.

A specific example of the first type of modification is acetylation of the N-terminal amino group of the B chain (B28, B25 or B23) or the modification of a tryptophan residue by addition of a formyl group.

Examples of the second type of modification are (i) replacement of the Trp moiety at B27 with glycine (Gly), (ii) replacement of the Pca moiety at B1 with glutamine (Gln) or glutamic (Glu) and (iii) replacement of Met moiety at A2 with norleucine (Nle).

An example showing both types of modification is replacement of the Pca moiety at B1 with N-acetylglutamine.

The invention in this aspect also includes relaxin analogues formed from natural or shortened B and/or A chains modified in accordance with the invention as described above.

The invention, in its various aspects, is further described and elucidated in the following examples.

EXAMPLE 1

(a) Starting Materials

The three structural variants of native relaxin (A22B28, A22B29 and A22B31) were isolated from pregnant pig ovaries and purified according to the procedure described by Sherwood and O'Byrne (10). Purified A22B31 peptide was also prepared by the method of Walsh and Niall (11).

(b) Separation of insulin and relaxin chains by S.sulfonation.

The A and B chains of porcine insulin were separated by S-sulfonation and purified according to the procedure previously described (13). For the S-sulfonation and separation of relaxin chains, the following modification to this procedure was used. Native porcine relaxin (A22B29, 19.8mg) was dissolved in 4ml tris buffer (0.05M, pH 8.3). To this solution was added sodium sulfite (28mg) and sodium tetrathionate (14mg) and the mixture stirred at 37°C. After 3.5 hours further sodium sulfite (14mg) and sodium tetrathionate (7mg) were added and the mixture stirred at 37°C for an additional 3 hours and then left to stand at room temperature overnight. The precipitated B-chain S-sulfonate was separated from A-chain S-sulfonate by centrifugation at 3000 rpm for 10 minutes. The B-chain sulfonate was partially purified by dissolving in dilute ammonia solution (5 ml) and precipitating at pH5 by the addition of glacial acetic acid, followed by centrifugation. This procedure was repeated twice. The precipitate was then redissolved in dilute ammonia solution and lyophilized to yield 11mg of B29 S-sulfonate. The supernatant solutions obtained from the above procedure were combined and dialyzed for 2 days against distilled water and then lyophilized to yield 6.3 mg of S-sulfonated A-chain.

The above procedure was also used to separate the A and B chains of porcine relaxin A22B28 and A22B31.

The time course of S-sulfonation for relaxin and insulin peptides was followed by titration with

p-chloromercuriobenzoic acid using procedures previously described (17).

When required, the S-sulfonated relaxin A-chain was further purified by ion exchange chromatography on DEAE-Sephadex A25 in tris-HCl buffer pH 8.5 using a sodium chloride gradient from 0 to 0.5M. Attempts to purify S-sulfonated relaxin B-chain by ion-exchange chromatography generally resulted in a complete loss of peptide by adsorption to the column. A reasonably satisfactory purification of B-chain was achieved by dissolving the S-sulfonated mixture in 8M urea formic acid pH 3.0 and applying the sample to a Dowex 5DWX2 ion-exchange column and eluting with a stepwise sodium chloride/urea gradient. Under normal conditions one would expect the S-sulfonated B-chain to elute first from the column. In practice, the B-chain adsorbed strongly to the Dowex and allowed the S-sulfonated A-chain to be eluted first. Purified S- sulfonated B-chain could be recovered, although in poor yield, by stripping the column with dilute ammonia solution at pH 9.5. Following dialysis and a precipitation-washing cycle at pH 9 and pH 3.8, S-sulfonated B-chain was obtained free from A-chain and sodium sulfite.

The S-sulfonated chains of relaxin were characterized by electrophoresis on cellulose acetate paper in phosphate buffer pH 7.8 (2000 V. for 20 minutes). The peptide spots were visualized by oxidation.

EXAMPLE 2

Synthesis of Porcine Relaxin A Chain

The 22-peptide chain representing the sequence of porcine relaxin A chain was assembled on a phenylacetamidomethyl - 1% cross linked polystyrene resin support (16) using standard solid-phase procedures (12). The amino acid side-chain functional groups were protected as follows: serine and threonine as the O-benzyl ether, aspartic and glutamic acids as the benzyl ester, arginine as the p-toluenesulfonyl-, lysine as the 2-chlorocarbobenzoxy- and cysteine as the p-methoxybenzyl-derivatives respectively. The completed peptide was cleaved from the resin support with hydrogen fluoride in the presence of anisole (10%) for 30 minutes at 0°C. Exposure of the peptide to hydrogen fluoride was minimized to prevent formation of the glutamic acid anisole adduct. The crude peptide was reduced with dithiothreitol in 6M guanidine hydrochloride and applied to a BioGel P10 column in 30% acetic acid. Further purification was by ion-exchange chromatography on SP-Sephadex with a pyridine-acetic acid gradient pH 2.5 to pH 5.0. The major peak from the SP-Sephadex column was S-Sulfonated (15) and applied to DEAE-Sephadex. The S-Sulfonated peptides were eluded with a linear gradient of NaCl to 0.5 M in tris-buffer (0.05M), pH 8.3 (Figure 4(a)). Relaxin A (1-22) SSO_3^- eluted at a conductivity of 11 to 13 mS and was characterized following dialysis by amino acid analysis, HPLC (see Figure 4(d)) and paper electrophoresis. The overall yield of purified Relaxin A (1-22) SSO_3^- was generally of the order of 10% based on the initial amount of crude peptide.

EXAMPLE 3

Synthesis of Relaxin A Chain Analogue

In a separate synthesis, using the general procedures described in Example 2, an analogue of the relaxin A-chain was prepared wherein the methionine at position 2 was replaced with the isosteric norleucine residue.

EXAMPLE 4

Synthesis of Porcine Relaxin B-Chain

Initially, the synthesis of CMa' or B(1-29) was attempted using the same general strategy outlined for the A-chain synthesis in Example 2. The crude B(1-29) peptide exhibited unusual solubility characteristics. Although the crude peptide dissolved completely in dithiothreitol-guanidine-30% acetic acid, the vast majority of the peptide was lost by adsorption or precipitation following gel filtration on BioGel or Sephadex. The B-chain peptide was soluble above pH 5.5 but precipitated out of solution on ion-exchange columns when eluted with a salt gradient. The salting-out or adsorptive effect of the synthetic B-chain occurred whether the peptide was in the reduced or S-Sulfonated form and severely hampered attempts to purify the peptide by conventional chromatography. Adequate purification of the synthetic B-chain could only be effected by precipitating the peptide at pH 5 and washing away the soluble impurities. A marked improvement in the solubility of the relaxin B-chain was noted when the B(1-29) peptide (CMa') was converted to B(1-28) (CMB) by removal of the carboxyl terminal arginine residue with Cpase B. The purification scheme

used for the synthetic S-Sulfonated relaxin B-chain, which resulted in a 15 - 20% yield based on the initial crude peptide, is outlined in Figure 2.

A convenient method for monitoring the purification of the synthetic relaxin A and B chains was found to be paper electrophoresis on cellulose acetate in phosphate buffer at pH 7.8.

EXAMPLE 5

(a) Synthesis of Relaxin B Chain Analogues

The B chain analogues set out in Table 1 below were prepared using the general procedures described in Example 2. Figures 4(b) and 4(c) show the results obtained in the first and final purification stages respectively for the synthetic S-sulfonated B(1-25) chain.

(b) Synthesis of [Gly^{27}] relaxin B-chain Analogue

The glycine-27 analogue of porcine relaxin B-chain in which the tryptophan which normally occurs at position 27 was replaced by glycine, was synthesized by the general procedures described in Example 2. The other tryptophan at position 18 in the porcine B-chain sequence was retained.

A modification of the procedures described in Example 2 was the addition of 0.2% indole to the acidic α-amino deprotection reagent following incorporation of the tryptophan-18 residue into the synthetic chain.

Following cleavage of the assembled peptide from the solid support the crude peptide was purified by gel filtration, ion exchange chromatography and preperative HPLC. In contrast to the porcine relaxin B(1-28) peptide, the reduced and S-sulfonated [Gly^{27}] analogue peptide did not adsorb to the chromatography media and could be effectively purified by these methods.

Circular dichroism analysis of the [Gly^{27}] analogue peptide gave spectra characteristic of a random coil configuration. This is in contrast to the native and synthetic relaxin B(1-28) peptide which were at least 90% of beta structure. These results suggest that the conformational and solubility characteristics of relaxin B-chain peptides are related to hydrophobic interactions involving the tryptophan residue at position 27.

The synthetic [Gly^{27}] porcine relaxin B(1-28) peptide was combined with synthetic porcine relaxin A-chain according to the procedures described in Example 6 (below). The chain combination mixture was biologically active in the <u>in vitro</u> rat uterine contractility assay and the <u>in vivo</u> guinea pig inter pubic ligament assay.

EXAMPLE 6

(a) <u>The recombination reaction</u>

Initially the S-sulfonated relaxin A and B chains were recombined using conditions previously found to be optimal for the recombination of insulin chains (18). As the recombination yields obtained for relaxin were

low using this general procedure (of the order of 1 to 2%), we investigated the solubility characteristics of the relaxin A and B chains, the influence of chain length of the relaxin B chain, the effect of the addition of urea, sodium chloride or dioxane to the recombination mixture, the ratio of A and B chains and the time of oxidation. The conformation of the relaxin A and B chains in solution and the structural changes occurring during chain combination were followed by circular dichroism spectroscopy (19).

(b) Preferred conditions for the recombination of porcine Relaxin A and B chains

S-sulfonated relaxin A chain (1.0 mg) and S-sulfonated relaxin B chain (1.0 mg) were dissolved in 0.05M tris-HCl buffer pH 8.3 (0.2 ml) in a stoppered test tube and the solution cooled to 0°C in an ice bath. A solution of mercaptoethanol in water (1.4N) was adjusted to pH8 by the dropwise addition of dilute ammonia solution and 0.12ml added to the solution of relaxin chains. The mixture was agitated and degassed three times with oxygen free nitrogen and placed in a 37°C water bath for 5.5 minutes. The solution was then adjusted to pH 4.5 with 30% acetic acid and the peptide chains precipitated by the addition of acetone (4 ml). The mixture was cooled to 0°C for 5 minutes then centrifugated. The precipitate was then wasted further with acetone (4 ml), ethyl acetate (4 ml) and finally petroleum ether (4 ml) and dried in vacuo. The precipitate was then dissolved in degassed water (0.2 ml) previously brought to pH 10 with the addition of 0.5N sodium hydroxide. To this solution was added 0.05M glycine buffer pH 10.6 (0.4ml) and sodium

chloride to give a final concentration of 1M and the mixture agitated and allowed to stand for 3 days at 4°C. The reaction mixture was centrifuged if necessary, and the peptide content in the supernatant measured by UV absorption at 280 nm prior to the assessment of the biological activity.

(c) Characterization of recombined peptide chains.

The recombination of the relaxin peptide chains was monitored by HPLC and radioimmunoassay using an antibody raised to native porcine relaxin A22B29 and I125 Bolton-Hunter labelled A22B29 tracer (22). The biological activity of the recombined products was assessed using the rat uterine contractility assay (14).

The biological activity of the synthetic relaxin peptides was tested in the rat uterine contractility assay (14).

Table 1 shows the actual combination yields (measured by bioassay) obtained for various combinations of the natural (N) and synthetic (S) A and B chains and various synthetic analogues. Column 3 shows the actual combination yields (based on the B chain). Column 4 shows the yields recalculated on the basis of 100% yield for the recombined natural A22 and B28 chains.

The following points are noted.

(i) The recombination yield for the synthetic A22 B28 chain is very much lower than that for the natural

chain. This is attributed to a lack of homogeneity in the synthetic peptides.

(ii) Recombination yields for the synthetic peptides are increased by shortening the B chain to 25 residues and/or by replacement of certain residues in the B and/or A chains.

(iii) Biological activity is retained even when the A and B chains are shortened at both termini.

TABLE 1

A-Chain	B-Chain	Recombination Yield (by BIOASSAY)	
NA (1-22)	NB (1-28)	25%	100%
SA (1-22)	SB (1-28)	0.7	3
SA (1-22)	SB[GLU^{1}](1-28)	2.1	8
SA (1-22)	SB[acetyl GLU^{1}](1-28)	2.1	8
SA (1-22)	SB (1-25)	5.0-7.0	24
SNle^{2}A (1-22)	SB (1-28)	1.5	6
SNle^{2}A (1-22)	SB[GLU^{1}](1-28)	2.5	10
SNle^{2}A (1-22)	SB[acetyl GLU^{1}](1-28)	1.6	6
SNle^{2}A (1-22)	SB (1-25)	4.0	16
SA (1-22)	SB (1-28)NH_2	1.7	7
SA (1-22)	SB (1-23)NH_2	0.8-2.0	6
SA (1-22)	SB[N-acetyl](4-23)NH_2	2.4	10
SA (4-22)	SB[acetyl GLU^{1}](1-28)	1.0	4
SA (4-22)	SB (1-25)	2.4	10
SA (4-22)	SB (1-23)NH_2	1.3	5
SA (4-22)	SB[N-acetyl](4-23)NH_2	1.3	5
SA (1-22)	SB[Gly^{27}] (1-28)	3.0	12
SA (1-22)NH_2	SB[Gly^{27}] (1-28)	2.4	10
SA (7-22)	SB[N-acetyl] (4-23)NH_2	0	0

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for the synthesis of relaxin or modified forms or analogues thereof, which comprises the steps of reducing a mixed solution of the S-sulfonated A and B peptide chains, or modified forms or analogues thereof; precipitating the reduced peptides with a non-reactive, neutral, organic, water-miscible solvents, washing the mixed peptides; oxidizing the mixed peptides in the presence of a non-reactive, water soluble, inorganic salt; and isolating the relaxin thus produced.

2. A method as claimed in Claim 1, wherein said peptide chains are porcine relaxin peptide chains.

3. A method as claimed in Claim 1 or Claim 2, which comprises the steps of:

separately preparing or isolating the S-sulfonated A and B peptide chains;

forming a mixture of the S-sulfonated A and B peptides;

reducing the mixture at pH of ~~about~~ 7 to ~~about~~ 9 under a non-reactive atmosphere for at least 6 minutes;

adjusting the pH of the reduced mixture to 4.5 to 5.5;

adding said non-reactive, neutral, water miscible solvent to the mixture to precipitate the mixed peptides;

washing the mixed peptides with a suitable solvent to remove the reducing agent;

oxidizing the mixed peptides at a pH of ~~about~~ 9.5 to ~~about~~ 11 for ~~about~~ 48 to 72 hours at a temperature of ~~about~~ 0° to 10°C in the presence of said

non-reactive water soluble inorganic salt in a concentration of at least 0.1M.

4. A method as claimed in Claim 3, wherein said non-reactive atmosphere is nitrogen.

5. A method as claimed in any one of Claims 1 to 4, wherein said neutral, organic, water-miscible solvent is acetone, and said non-reactive water soluble inorganic salt is sodium chloride.

6. A method as claimed in any one of Claims 1 to 5, wherein a shortened form of one or both of the A and B chains is used.

7. A method as claimed in any one of Claims 1 to 5, wherein at least one amino-acid in one or both of the A and B chains is chemically modified prior to combination.

8. A method as claimed in Claim 7, wherein the chemical modification comprises the addition of a protective group to a free amino group, and the protective group is optionally removed following combination.

9. A synthetic porcine relaxin analogue consisting of the A(1-22) and B(1-25) peptide chains.

10. A synthetic porcine relaxin analogue consisting of A (1-22) and B [GLU^1](1-28) peptide chains.

11. A synthetic porcine relaxin analogue consisting of the A (1-22) and B [acetyl GLU^1](1-28) peptide chains.

12. A synthetic porcine relaxin analogue consisting of the A[Nle^2](1-22) and B[GLU^1](1-28) peptide chains.

13. A synthetic porcine relaxin analogue consisting of the A[Nle^2](1-22) and B[acetyl GLU^1](1-28) peptide chains.

14. A synthetic porcine relaxin analogue consisting of the A[Nle^2](1-22) and B (1-25) peptide chains.

15. A synthetic porcine relaxin analogue consisting of the A (1-22) and B (1-28)NH_2 peptide chains.

16. A synthetic porcine relaxin analogue consisting of the A (1-22) and B (1-23)NH_2 peptide chains.

17. A synthetic porcine relaxin analogue consisting of the A (1-22) and B[N-acetyl](4-23)NH_2

peptide chains.

18. A synthetic porcine relaxin analogue consisting of the A (4-22) and B[acetyl GLU^1](1-28) peptide chains.

19. A synthetic porcine relaxin analogue consisting of the A (4-22) and B (1-25) peptide chains.

20. A synthetic porcine relaxin analogue consisting of the A (4-22) and B (1-23)NH_2 peptide chains.

21. A synthetic porcine relaxin analogue consisting of the A (4-22) and B[N-acetyl](4-23)NH_2.

22. A porcine relaxin analogue which has been modified by one or more of the following procedures:

(a) the addition of one or more amino-acids to one or both of the natural A and B chains;

(b) the deletion of one or more amino acids from the natural B and/or A peptide chains;

(c) the chemical modification of one or more amino acids

(d) the replacement of at least one of the natural amino-acids in one or both of the A and B chains with a different amino acid of the natural B and/or A peptide chains;

with the proviso that the thus modified porcine relaxin analogues possess porcine relaxin activity.

23. An analogue as claimed in claim 22 wherein

the chemical modification comprises the addition of a protective group to a free amino group.

DATED this 26th day of February, 1987

HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND MEDICINE

by its Patent Attorneys

DAVIES & COLLISON

A CHAIN

1 5 10

H-ARG-MET-THR-LEU-SER-GLU-LYS-CYS-CYS-GLN-VAL-GLY-CYS

15 20 22

ILE-ARG-LYS-ASP-ILE-ALA-ARG-LEU-CYS-OH

B CHAIN

1 5 10

PCA-SER-THR-ASN-ASP-PHE-ILE-LYS-ALA-CYS-GLY-ARG-GLU

15 20 25

LEU-VAL-ARG-LEU-TRP-VAL-GLU-ILE-CYS-GLY-SER-VAL-SER

28 29 31

TRP-GLY-ARG-THR-ALA-OH

FIGURE 1

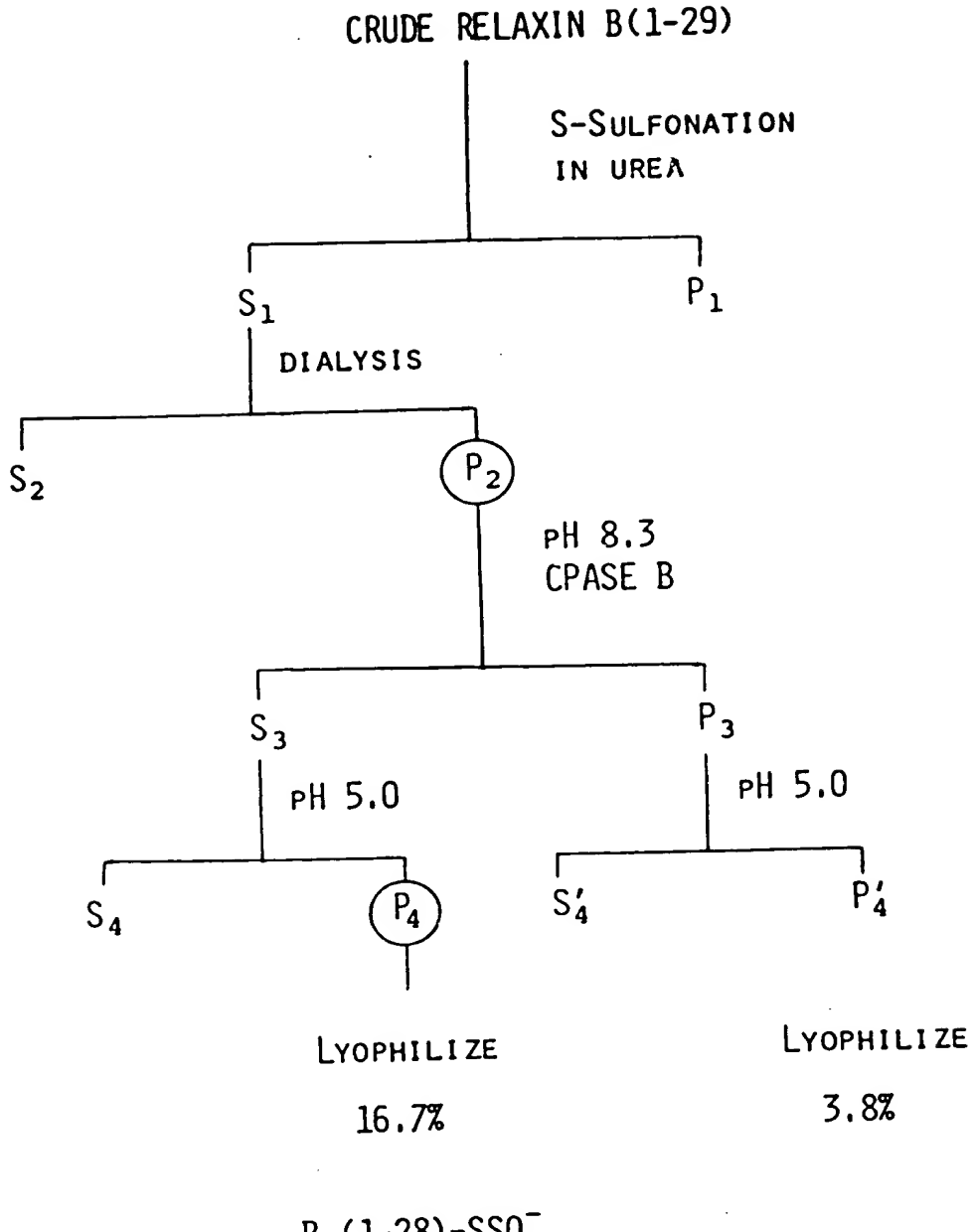

CRUDE RELAXIN B(1-29)
S-SULFONATION
IN UREA
S_1
P_1
DIALYSIS
S_2
P_2
pH 8.3
CPASE B
S_3
P_3
pH 5.0
pH 5.0
S_4
P_4
S'_4
P'_4
LYOPHILIZE
16.7%
LYOPHILIZE
3.8%
B-(1-28)-SSO_3^-

FIGURE 2

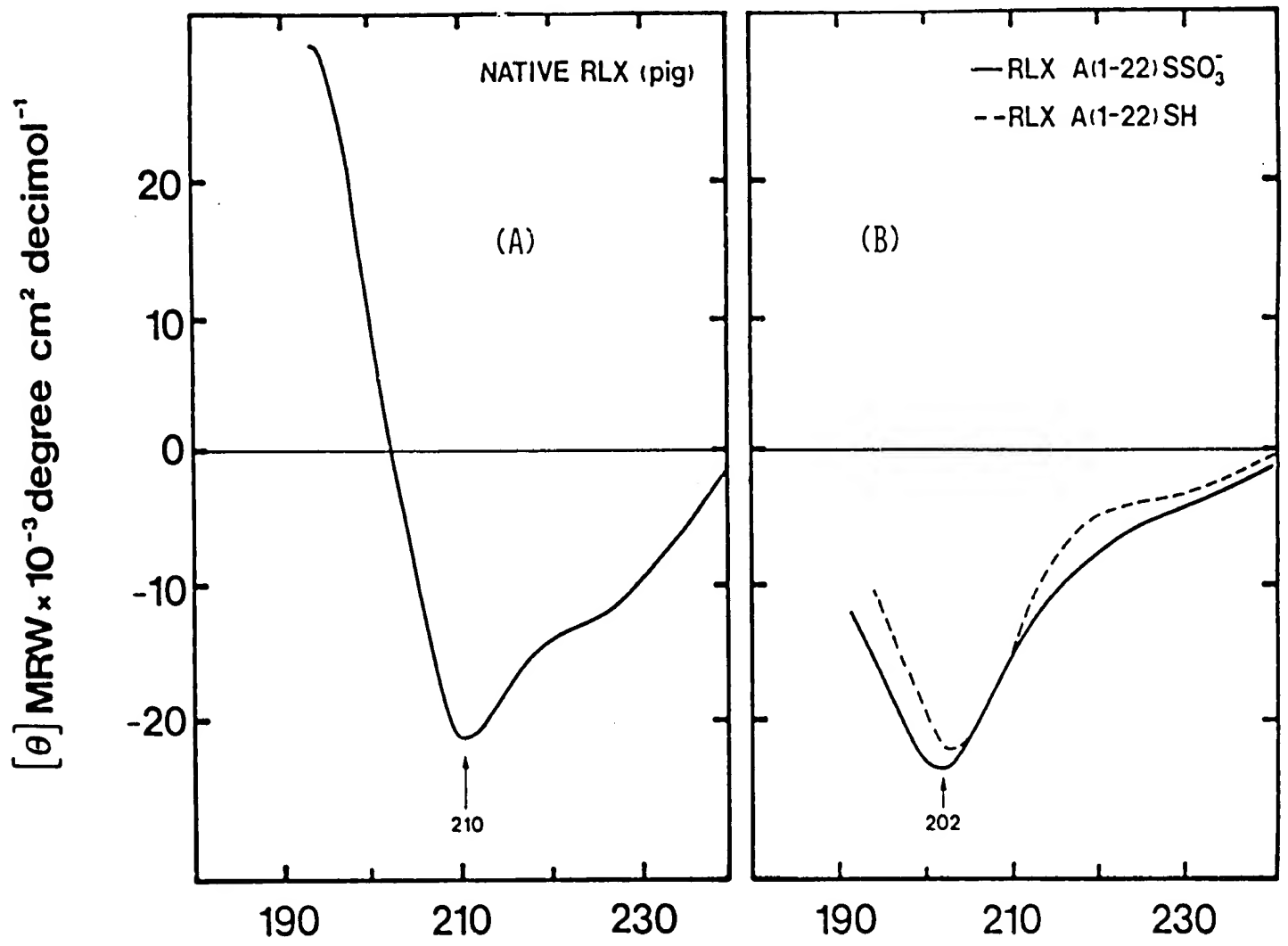

NATIVE RLX (pig)
(A)
(B)
—RLX A(1-22)SSO$_3^-$
--RLX A(1-22)SH
[θ]MRW × 10^{-3} degree cm^2 decimol^{-1}
20
10
0
-10
-20
210
202
190
210
230
190
210
230
Wavelength nm

FIGURE 3(1)

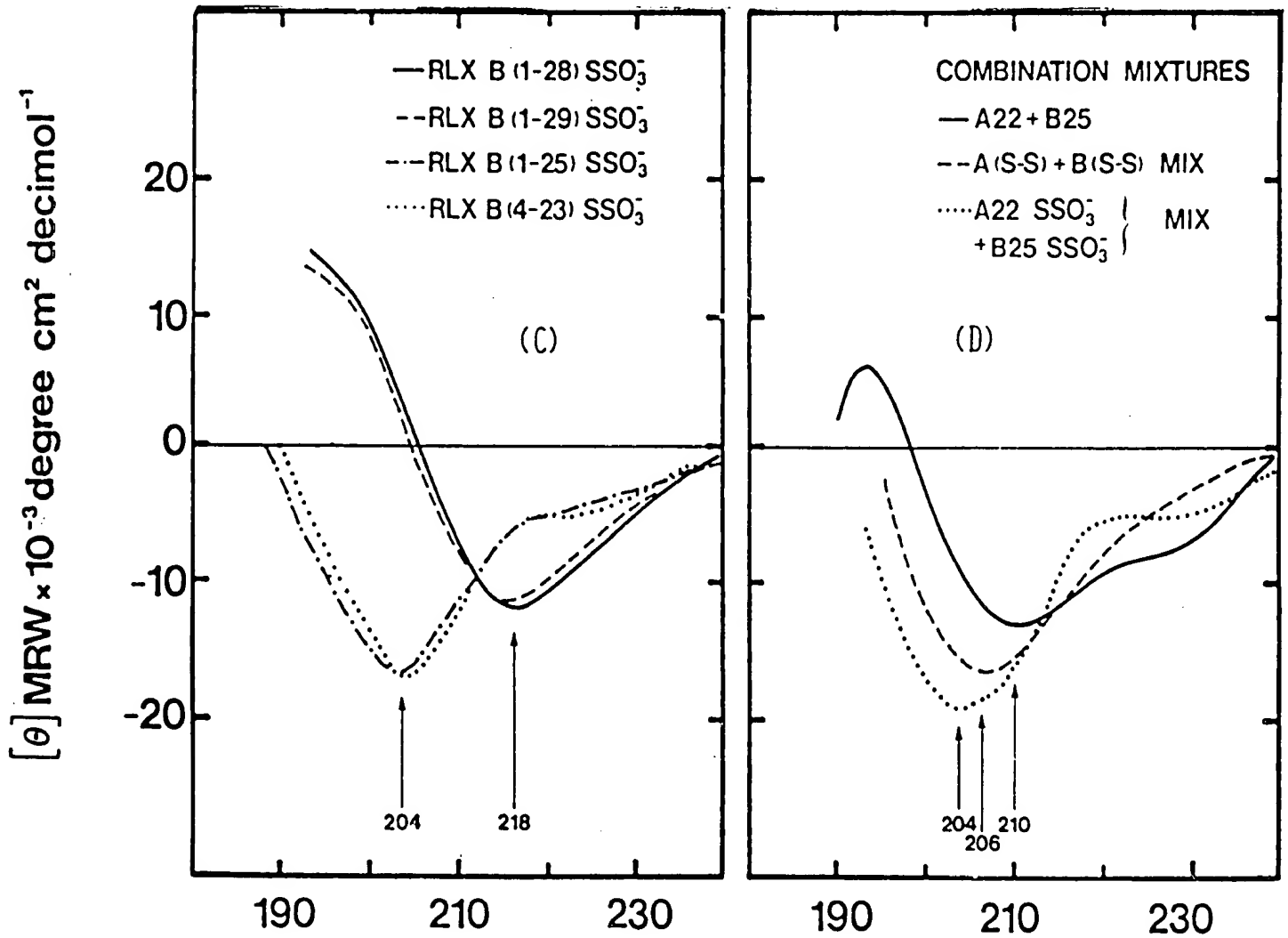

—RLX B (1-28) SSO3-
--RLX B (1-29) SSO3-
-·-RLX B (1-25) SSO3-
·····RLX B (4-23) SSO3-
(C)
COMBINATION MIXTURES
— A22 + B25
--A (S-S) + B (S-S) MIX
·····A22 SSO3- + B25 SSO3- MIX
(D)
[θ] MRW × 10-3 degree cm2 decimol-1
20
10
0
-10
-20
204
218
204
206
210
190
210
230
190
210
230
Wavelength nm

FIGURE 3(2)

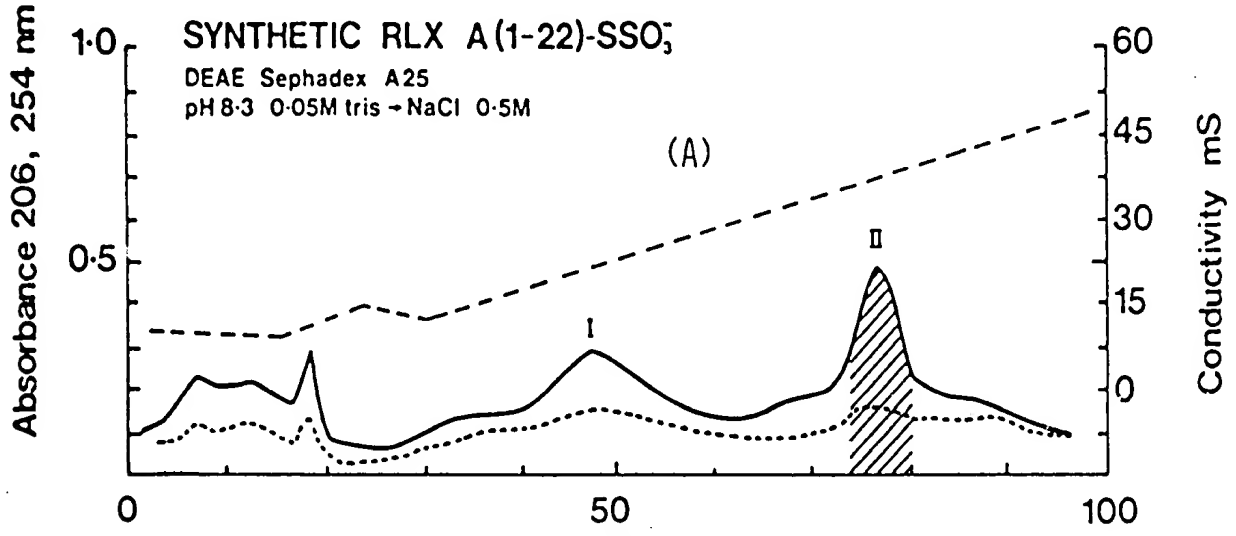
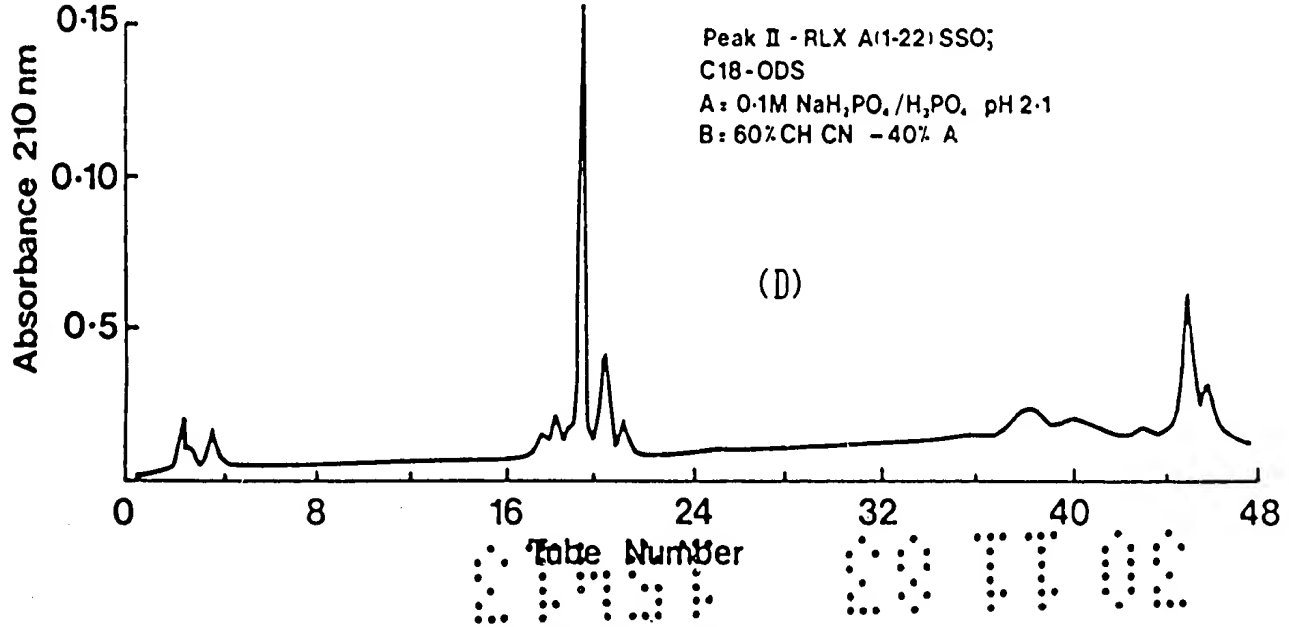
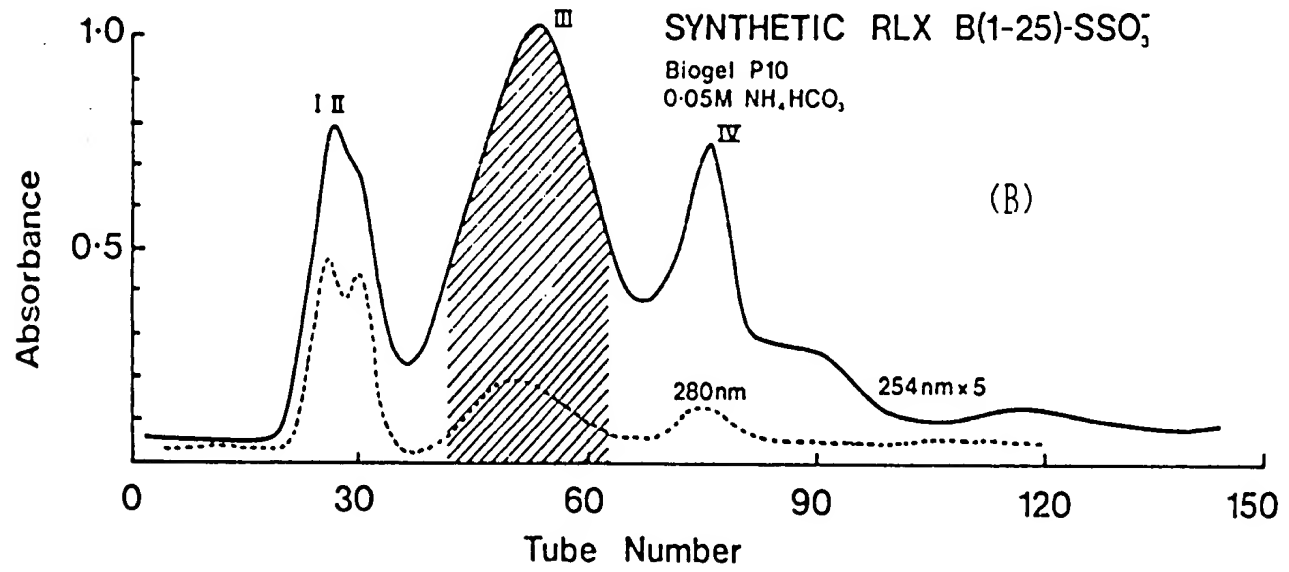

SYNTHETIC RLX A(1-22)-SSO3
DEAE Sephadex A25
pH 8·3 0·05M tris → NaCl 0·5M
(A)
Absorbance 206, 254 nm
Conductivity mS
1·0
0·5
60
45
30
15
0
I
II
0
50
100
Tube Number

FIGURE 4(1)


Peak II - RLX A(1-22)SSO3
C18-ODS
A: 0·1M NaH2PO4/H3PO4 pH 2·1
B: 60% CH CN - 40% A
(D)
Absorbance 210 nm
0·15
0·10
0·5
0
8
16
24
32
40
48
Tube Number


SYNTHETIC RLX B(1-25)-SSO3-
Biogel P10
0·05M NH4HCO3
(B)
Absorbance
1·0
0·5
I II
III
IV
280nm
254nm×5
0
30
60
90
120
150
Tube Number

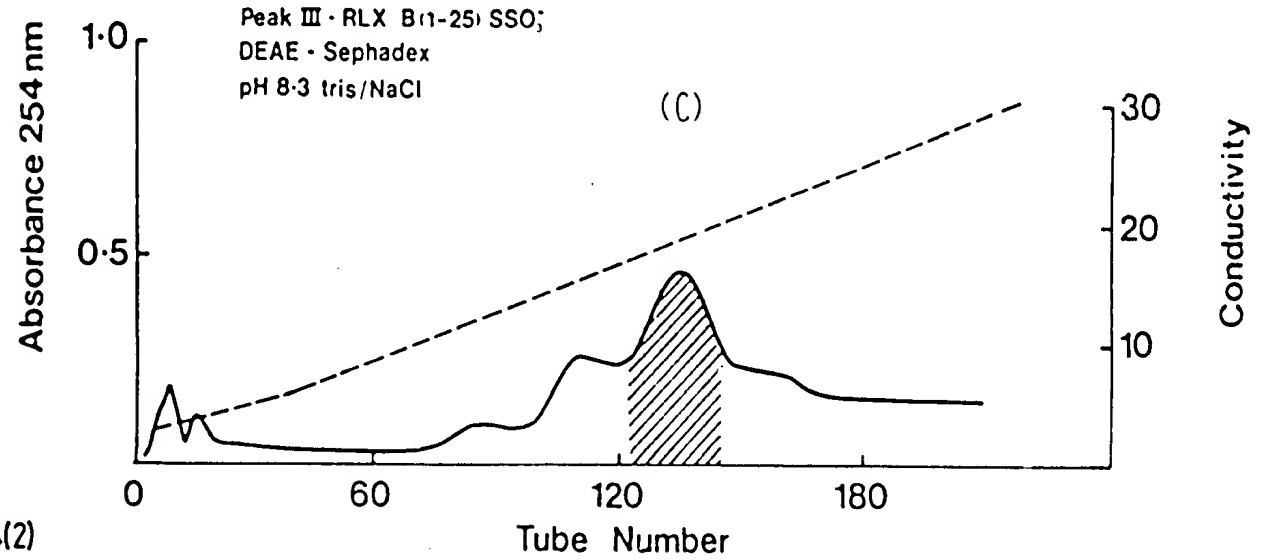

Peak III - RLX B(1-25) SSO3-
DEAE - Sephadex
pH 8·3 tris/NaCl
(C)
Absorbance 254 nm
1·0
0·5
30
20
10
Conductivity
0
60
120
180
Tube Number

FIGURE 4(2)

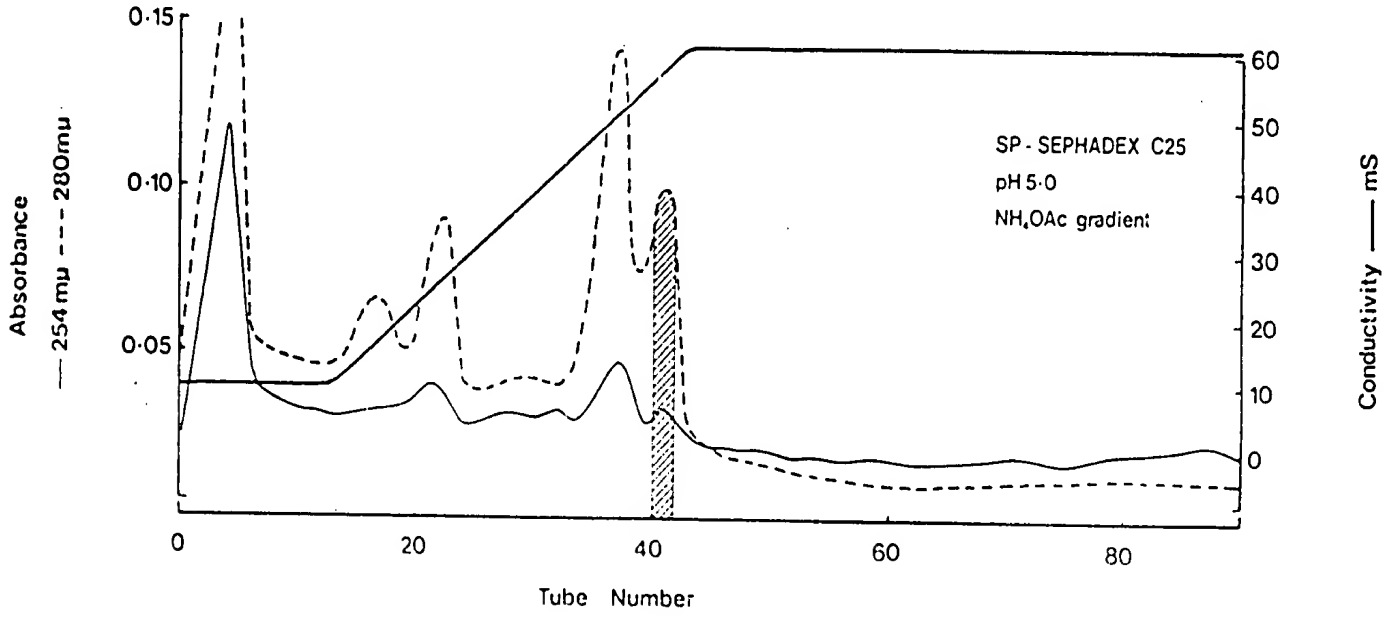

0·15
0·10
0·05
Absorbance
— 254mμ - - - 280mμ
0
20
40
60
80
Tube Number
60
50
40
30
20
10
0
Conductivity —— mS
SP - SEPHADEX C25
pH 5·0
NH₄OAc gradient

FIGURE 5

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